New blood vessels can be induced to invade ischemic skeletal muscle

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Purpose: **Despite intense investigation of angiogenesis, little effort has been made to exploit this phenomenon in ischemic tissue. The few studies on this topic have focused primarily on the development of collateral arteries in existing arterial beds. A previous study showed that a perfused muscle flap transposed to an ischemic limb formed vascular connections, which were demonstrated with angiography, between the arteries of the flap and the limb vasculature. Microsphere studies suggested that these vascular connections increased the resting perfusion of the ischemic limb. This study is designed to confirm histologically that such connections develop and to quantitate the number and dimensions of these new vessels.**

Methods: **Through a midline laparotomy, the right common iliac artery was ligated and divided in 18 male New Zealand white rabbits. An abdominal-wall muscle flap based on the left inferior epigastric artery was transposed to the right thigh. On the seventh day, contrast dye was injected into the flap artery of eight rabbits and an arteriogram was obtained. The tissue of the remaining rabbits was perfusion-fixed at 3 days (n = 2), 7 days (n = 4), and 14 days (n = 4). Thin sections of the flap–thigh muscle interface were stained with hematoxylin and eosin and for α-actin and proliferating cell nuclear antigen and were examined microscopically.**

Results: **An arteriogram confirmed vascular connections between the flap and the native limb circulation in seven of the eight rabbits. Histologic evaluation of the flap–thigh muscle interface showed no new vessels on the third day. On the seventh day, 6.8 ± 4.8 new vessels (positive α-actin staining, red blood cells in lumen) were seen per 40× field; the vessels averaged 10.2 ± 5.2 μm in diameter. On the fourteenth day, there were 7.3 ± 3.8 vessels per 40× field ($p = 0.46$), but the vessel diameter increased to 20.7 ± 10.6 μm ($p = 0.013$). Proliferating cell nuclear antigen staining confirmed that these were proliferating vessels.**

Conclusion: **Within seven days, new vessels that were more mature than capillaries (stained for α-actin, a smooth muscle cell protein) formed between the flap and the thigh muscle. These new connecting vessels continue to enlarge in diameter between 7 and 14 days, but the stimulus to form new vessels appeared to decline or disappear before the fourteenth day. Attempts to sustain this phenomenon with angiogenic factors are underway. (J Vasc Surg 1996;24:534-44.)**

Current techniques that are used to salvage ischemic tissues rely on the reconstruction of macroarteries. There exists, however, a subgroup of patients who have ischemic organs or limbs and in whom no patent macroarteries are available for reconstruction. Currently, these patients are treated with ablation rather than reconstruction: removal of the ischemic organ or limb amputation.

Over the years, there have been a few efforts to indirectly revascularize ischemic organs and limbs. These efforts include the simple insertion of the internal mammary artery into a hole made in ischemic myocardium[1,2] or the transfer of omental pedicles onto ischemic limbs.[3-6] Unfortunately, these methods were either unsuccessful or successful only in patients who had narrow categories of disease (e.g., omental flaps worked well in patients who had Buerger's disease, but failed in patients who had atherosclerotic

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occlusive disease). Consequently, methods of indirect revascularization of ischemic tissues have not been clinically useful.

The phenomenon of angiogenesis—the development of new blood vessels—has been extensively studied in recent years. Much is now known about the mechanism and control of angiogenesis. The temporal and microanatomic stages of blood vessel development have been described.[7] Various peptides that control blood vessel development have been identified. Most of the investigations into angiogenesis, however, have been focused on methods to delay or halt blood vessel development in an effort to slow or prevent tumor growth and to decrease retinal neovascularization. Little attention has been given to exploiting angiogenesis in an attempt to revascularize ischemic tissue.

The recent advances in the understanding of the mechanism and control of angiogenesis provide an opportunity to reexamine the concept of indirect revascularization of ischemic tissues and to possibly enhance the innate biologic tendency for new blood vessels to develop. A previous study one of the authors (WCP) showed that a perfused muscle flap transposed to an ischemic limb formed vascular connections, which were demonstrated on angiographic examination, between the arteries of the flap and the limb vasculature.[8] Labeled microsphere studies suggested that these vascular connections increased the resting perfusion of the ischemic limbs.

In our study, with the use of a similar model, we reexamined the hypothesis that new vascular connections develop between a well-perfused muscle flap and the blood vessels of an underlying ischemic limb. This hypothesis was indirectly supported, by means of angiography and labeled microsphere studies, in a previous study.[8] Our current study was designed to provide direct histologic confirmation of the development of new blood vessels between the well-vascularized muscle flap and vessels of the underlying ischemic skeletal muscle. An attempt was made to quantitate the number and dimensions of the new vessels that formed. These new vessels were evaluated at three different points in time to assess their temporal development. This descriptive study was performed to gain insight into the phenomenon of the development of new vessels growing between healthy skeletal muscle and ischemic skeletal muscle and to develop techniques that will permit future studies of this phenomenon to be quantitative.

MATERIAL AND METHODS

Eighteen male New Zealand white rabbits weighing 2.5 to 3.5 kg were used as the study animals. The study design was approved by the Animal Use and Care Administrative Advisory Committee of the University of California, Davis, and the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* (NIH Publication #86-23, revised 1985) was followed throughout the study. General anesthesia was induced with 0.5 mg/kg acepromazine, 5 mg/kg xylazine, and 50 mg/kg ketamine administered by intramuscular injection. Normal saline solution was slowly infused through an intravenous line placed in a dorsal ear vein. Enrofloxacin 10 mg/kg was administered by intramuscular injection.

With the use of sterile technique, a midline laparotomy was performed and the right common iliac artery was ligated and transected. A previous study confirmed that this model consistently produces partial limb ischemia that persists for at least 17 days.[9] A muscle flap based on the left deep inferior epigastric artery was developed. This artery courses between the transversalis and internal abdominal oblique muscles, lateral to the rectus sheath. The flap was based at the origin of the left deep inferior epigastric artery, and the distal extent of the flap was determined by the terminal arborization of this artery. The flaps measured approximately 2 cm by 8 to 10 cm. After raising the flap, the abdominal muscles were reapproximated at the midline. The peritoneum was dissected off of the distal 2 cm of the flap. One milliliter of 10% fluorescein was administered by intravenous injection, and the flap was examined with a Wood's lamp. Any areas of the flap that were not phosphorescent were debrided.

A 3-cm longitudinal incision was made in the medial right thigh, and the deep fascia was dissected off the medial aspect of the gracilis and rectus femoris muscles. The flap was passed through a subcutaneous tunnel and sutured to the gracilis and rectus femoris muscles (Fig. 1). The skin incisions were closed, and the rabbits were allowed to recover.

On the seventh day after surgery, the rabbits again were anesthetized with the same agents as described above. Eight of the rabbits were killed with an overdose of ketamine and potassium chloride. The flap was transected at its base, just distal to the origin of the left deep inferior epigastric artery. The flap artery was cannulated with a blunt-tip 26-gauge needle, and an arteriogram was obtained by injecting 0.3 to 1.2 ml of sodium diatrizoate or sodium iothalamate into the flap artery, such that any contrast reaching the limb originated in the flap artery.

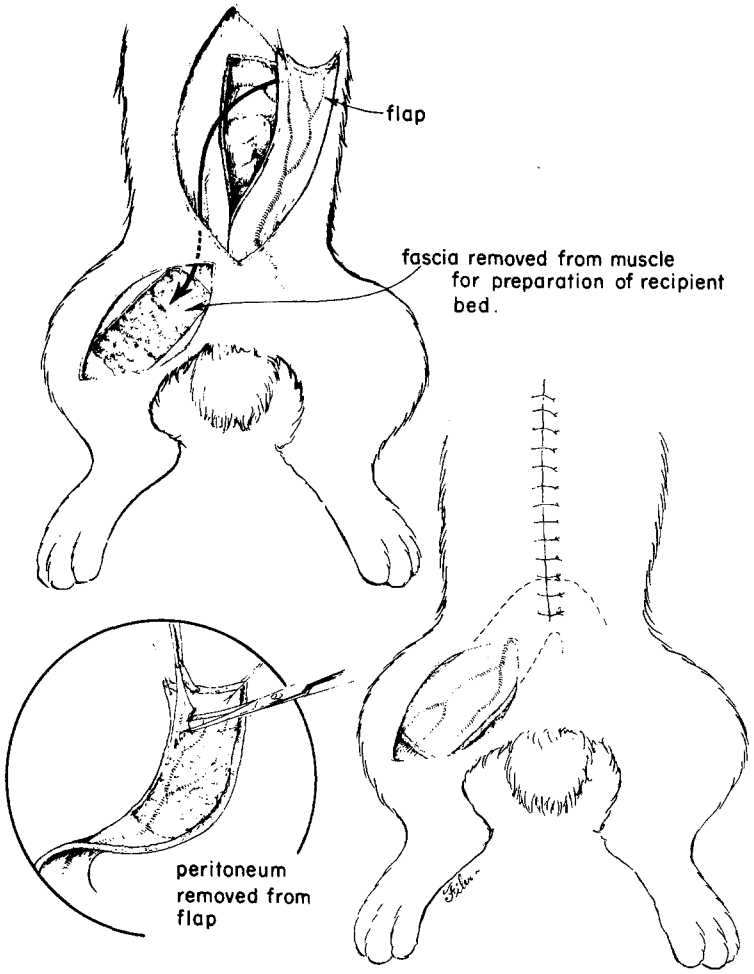

Fig. 1. After ligation of right common iliac artery, a muscle flap based on left deep inferior epigastric artery is transposed onto muscle of right thigh (From Pevec WC, Hendricks D, Rosenthal MS, Shestak KC, Steed DL, Webster MW. J Vasc Surg 1991;13:385-90).

In the remaining 10 rabbits, the muscle flap and underlying muscle were harvested for histologic evaluation. The harvest took place on the third day after surgery in two rabbits, on the seventh day after surgery in four rabbits, and on the fourteenth day after surgery in four rabbits. The rabbits were anesthetized with the same agents used in the initial operation. The infrarenal aorta was dissected and looped with silk ties. The right common carotid artery was cannulated with an 18-gauge catheter, and 2000 units of heparin were infused. After allowing the heparin to circulate for 2 minutes, the infrarenal aorta was ligated proximally and then cannulated distally with a 14- or 16-gauge catheter. The catheter was flushed with heparinized saline solution. The rabbit was exsanguinated through the carotid catheter, and its death then was ensured with an overdose of ketamine and potassium chloride. The pelvis and hind-limb tissues were perfusion-fixed with 1000 ml of 10% formalin infused into the distal aorta at a pressure of 100 mm Hg. A block of tissue including the entire interface between the flap and the underlying muscle then was removed (Fig. 2) and placed in 10% formalin.

The tissue blocks were dehydrated and then embedded in paraffin. Thin sections (4 μm) were cut perpendicular to the flap–thigh muscle interface (Fig. 2). The sections were stained with hematoxylin and eosin with routine histologic techniques.

The sections also were immunostained for α-actin. Anti-α-actin antibody (Boehringer Mannheim Biotechnology, Indianapolis) was applied to the sections at 1:150 and 1:200 dilution; immunoperoxidase counterstaining was performed with standard techniques. This antibody only recognizes the α-actin isoform of smooth muscle cells. It does not react with

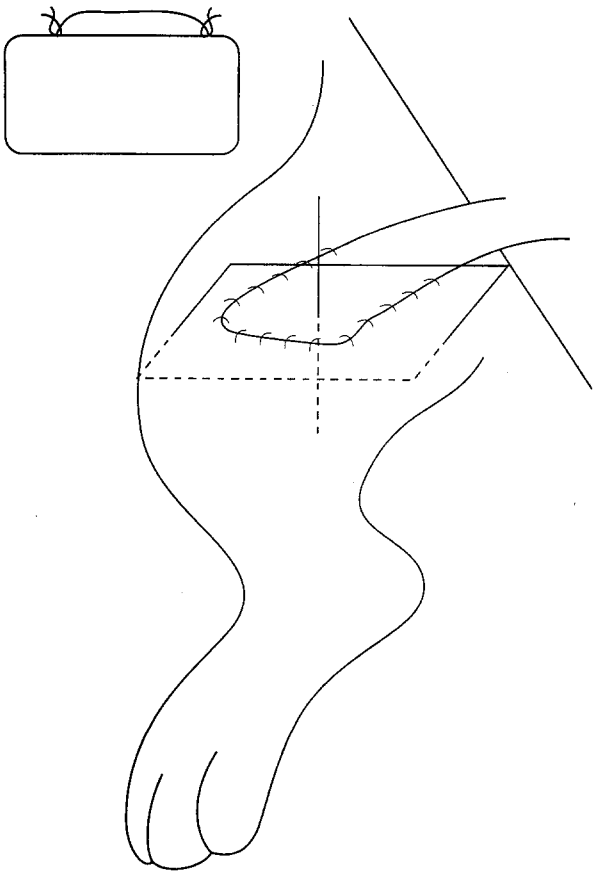

Fig. 2. Tissue block containing entire distal end of flap and underlying muscles of hind limb is sectioned perpendicular to flap-limb interface.

Fig. 3. Contrast injected into flap artery on seventh day after surgery opacified native macrocirculation of right hind limb.

striated cardiac and skeletal muscle cells. This antibody previously has been shown to effectively stain smooth muscle from human beings, cows, rats, mice, and chickens.

Immunostaining with proliferating cell nuclear antigen (PCNA; Dako, Carpenteria, Calif., and Oncogene Science, Uniondale, N.Y.) at a 1:50 dilution also was performed. This antibody stains for the nuclear material of proliferating cells. Positive staining indicates active cell division.

The stained slides were examined with light microscopy at 40× and 100× magnification. The slides were scanned, and when clusters of vessels were identified the field was centered on these vessels. Blood vessels were identified by tubular morphologic features with a single layer of endothelial cells, the presence of intraluminal red blood cells, and positive α-actin staining. With the fields so defined, the number of vessels per 40× field were counted by three separate observers; any variation in counts were adjudicated by these three observers. For each slide, the highest and lowest counts were discarded from further analysis.

An eyepiece micrometer was used to measure the diameter of the blood vessels. Only vessels cut on cross-section were measured; vessels cut tangentially were not measured. Computer-assisted image analysis was used to verify the micrometer measurements. Again, with each slide the highest and lowest values were discarded from further analysis.

Vessel counts and measurements were all performed by direct microscopic evaluation, alternating between 40× and 100× magnification to improve resolution. The counts, however, are reported as number of vessels per 40× field.

The vessel counts and vessel diameters were compared with the Mann-Whitney rank sum test for the comparison of nonparametric data. A p value less than 0.05 suggested statistical significance.

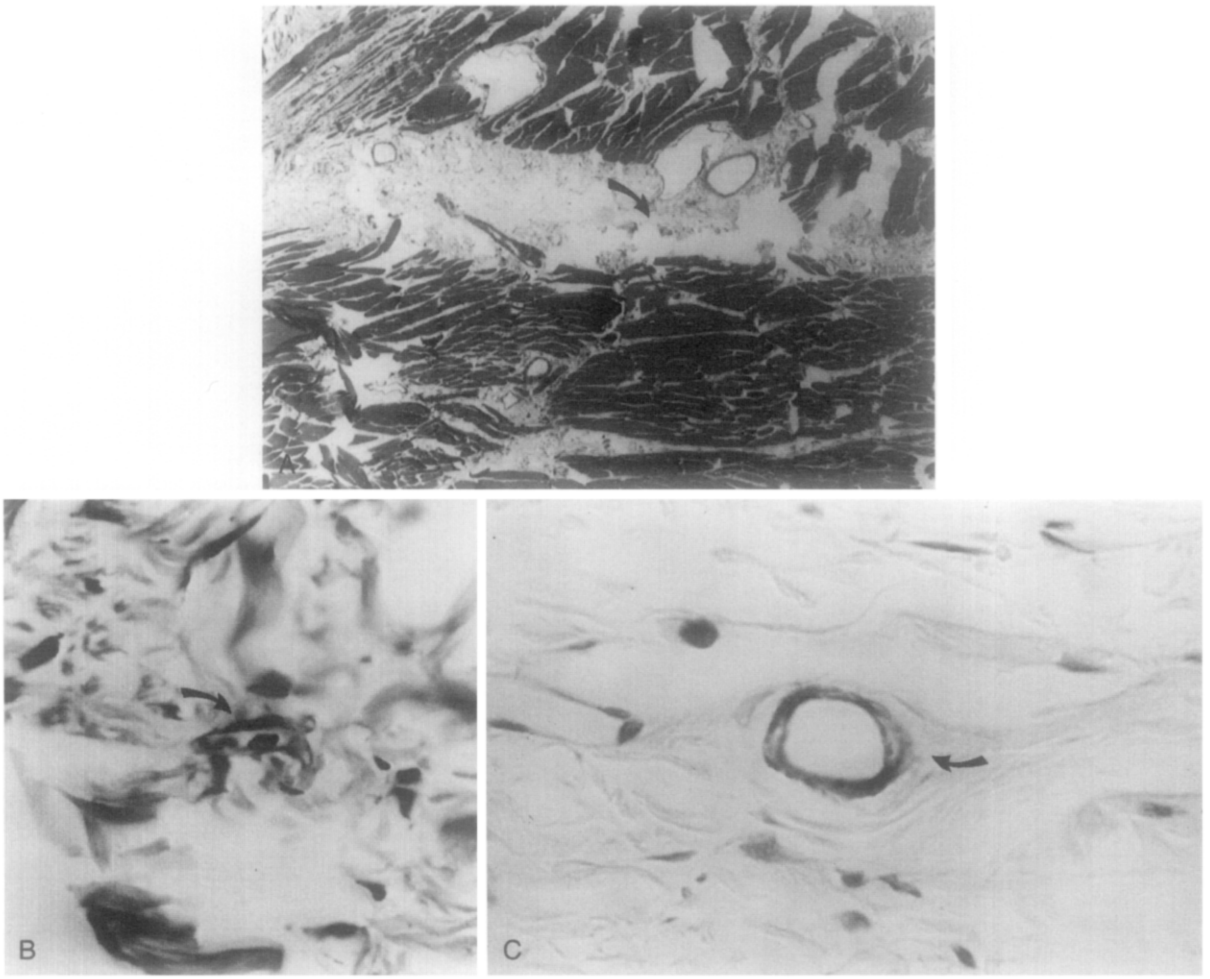

Fig. 4. A, Microvessels can be seen at flap-limb interface at low magnification (*small arrow;* hematoxylin and eosin stain). **B,** At 100× magnification, tubular morphologic features with endothelial cells and intraluminal erythrocytes can be seen (*large arrow*; hematoxylin and eosin stain). **C,** Microvessel clearly stains with antibody to α-actin *(arrow).*

RESULTS

Arteriographic examination on the seventh day after the operation confirmed the presence of vascular connections between the flap and the native hind-limb circulation in seven of eight rabbits (Fig. 3). The flap vessels were seen to opacify, as were several native macrovessels in the hind limb, when radiopaque contrast was injected directly into the divided flap artery.

Good sections were obtained from nine of the 10 rabbits that were examined for histologic evidence; on the seventh day after surgery, one of the four rabbits had extensive necrosis of the right thigh muscle, which precluded useful histologic evaluation. Effective immunostaining of the microvessels was achieved with α-actin and PCNA staining. No clusters of microvessels could be identified at the flap–hind-limb muscle interface on the third day after surgery. On the seventh day after surgery, 6.8 ± 4.8 (mean ± standard deviation) vessels were seen per 40× field, with a median of five and a range of three to 25 vessels per 40× field (Figs. 4 and 5, Table 1). The diameter of these vessels averaged 10.2 ± 5.2 μm, with a median diameter of 8 μm and a range of 4 to 20 μm.

On the fourteenth day after surgery, no significant change in the mean number of vessels was noted. There was an average of 7.3 ± 3.8 vessels per 40× field ($p = 0.46$, 14 days vs 7 days), with a median of six vessels and a range of two to 15 vessels per 40× field.

At 14 days, however, a significant increase in the mean diameter of the new microvessels had occurred. The average diameter was 20.7 ± 10.6 μm ($p = 0.013$, 14 days vs 7 days), with a median diameter of 22 μm and a range of 5 to 35 μm.

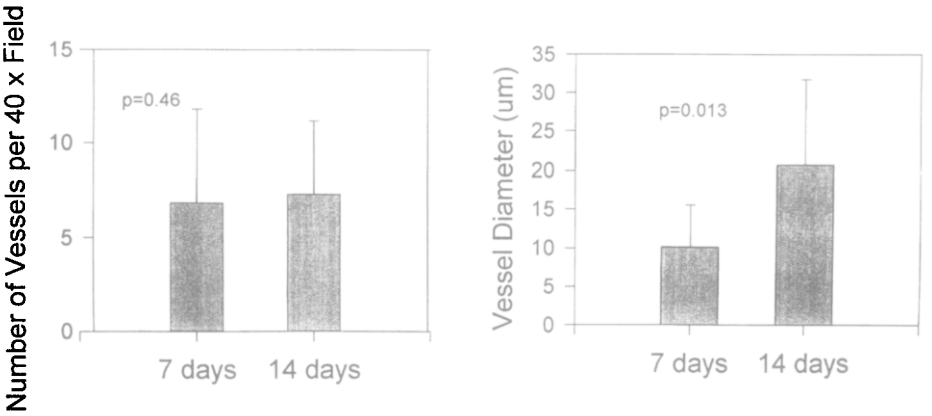

Fig. 5. Number and diameter of new vessels at 7 and 14 days.

DISCUSSION

This investigation confirms that new blood vessels develop to bridge the interface between adjacent healthy and ischemic skeletal muscle and that these new blood vessels provide a connection between the vasculature of the healthy muscle flap and that of the ischemic limb. The absence of clusters of microvessels at the flap-limb interface on the third day after surgery, with the clear presence of these microvessels on the seventh and fourteenth days after surgery, suggests that these clusters truly represent newly formed vessels. The positive staining of the microvessels with PCNA, which binds only to the nuclear material of proliferating cells, further confirms that these microvessel clusters are new, developing blood vessels. The arteriograms, which showed opacification of the native limb vasculature after the injection of radiopaque contrast into the flap artery, suggest that these new vessels form connections between the macrocirculation of the flap and that of the ischemic limb.

Tissue injury appears to be a strong stimulus for angiogenesis. It has been shown that damaged skeletal muscle can induce angiogenesis.[10] Endothelial cell–stimulating angiogenic factor has been isolated in rat skeletal muscle.[11] Basic fibroblastic growth factor (bFGF), a potent direct stimulus of angiogenesis, is thought to be released from endothelial basement membrane in response to injury.[12] Free muscle grafts implanted into well-vascularized recipient beds will develop vascular connections between the circulation of the grafted muscle and the recipient bed.[13,14]

Hypoxia is also a stimulus for angiogenesis. Macrophages have been shown to secrete angiogenic factors in the presence of hypoxia.[15] Vascular endothelial growth factor (VEGF), another potent direct stimulus to blood vessel formation, is induced by hypoxia.[16,17] In vitro, hypoxia and bFGF have been shown to act synergistically to increase VEGF production from vascular smooth muscle.[18]

The stimulus for angiogenesis in this model cannot be determined from this study. In previous unpublished experiments with this model, vascular connections were demonstrated on angiographic ex-

Table I. Number and diameter of new vessels at 7 and 14 days

	Vessel number		*Vessel diameter*	
	7 day	*14 day*	*7 day*	*14 day*
	3	2	4	5
	3	2	5	6
	4	3	5	8
	4	3	6	9
	5	4	7	11
	5	5	8	15
	5	5	9	18
	5	5	11	22
	5	6	14	26
	5	6	15	29
	5	7	18	30
	6	8	20	32
	6	8		32
	7	9		33
	8	10		35
	8	11		
	8	12		
	13	12		
	15	13		
		15		
Mean	6.8*	7.3*	10.2†	20.7†
Standard deviation	4.8	3.8	5.2	10.6
Range	3-25	2-15	4-20	5-35
Median	5	6	8	22

*$p = 0.46$.
†$p = 0.013$.

amination between the muscle flap and a limb with no previous arterial ligation. Thus although ischemia may stimulate angiogenesis, ischemia does not appear to be a requirement for angiogenesis in this model; local tissue injury alone is a sufficient stimulus for angiogenesis.

In this study, it was not possible to determine whether the new vessels grew from the flap to the ischemic limb, or from the ischemic limb to the flap. Intuitively, it would seem that the new vessels should invade the ischemic muscle from the healthy flap. However, in a model of free muscle fiber implantation into the cheek pouch of a hamster, Faulkner et al.[13] presented histologic evidence that suggested that the new vessels develop in the free muscle implant and invade the surrounding cheek pouch.

It is likely that the developing vessels at the interface between the flap and the ischemic limb are present on the third day after the operation but cannot be detected with α-actin staining. At this stage, the vessels most likely consist only of tubes of endothelium with no supporting smooth muscle and thus do not stain with antibodies to α-actin, a smooth muscle cell protein. By the seventh day, the vessels are more mature, with smooth muscle cells in the vessel wall, and the vessels exhibit actin staining. Several studies suggest that invasion of new blood vessels into various tissues or exogenous matrixes occurs within 2 to 7 days.[13,14,19-22] Thus the time course of angiogenesis in this model is consistent with that described by other investigators.

The new blood vessels in this study increased in size, but not in number, between the seventh and fourteenth days. This finding suggests that the stimulus for the new vessel development declines or disappears between the seventh and fourteenth days after surgery, whereas the stimulus for vessel enlargement persists. Although the explanation for this disappearance cannot be determined by our present study, changing degrees of muscle ischemia in the limb may play a role. A previous study of the model of common iliac artery ligation suggested that a gradual improvement in the perfusion of the experimental limb occurs with time, apparently as a result of the development of collateral circulation.[9] This collateral circulation, along with the improvement in local oxygen tension at the flap-limb interface that is caused by the presence of the newly developed microvessels, might decrease the hypoxic stimulus of angiogenesis. This hypothesis is consistent with that of metabolic control of angiogenesis, proposed by Adair et al.[12] The demands on the new microvessels to provide increased flow to the connections with native-limb vessels, however, would be expected to lead to a continued increase in the diameter of the new vessels. Such an explanation is only speculative; the current study was designed only to confirm and quantitate histologically the development of new blood vessels across the flap-limb interface in this model, not to elucidate the stimulus or control of this phenomenon. It is possible that the development of new vessels is a response to local tissue injury, and that this stimulus subsides with healing. Regardless of the innate mechanisms that control these phenomena, techniques that could be used to maintain the stimuli both for the development of new vessels and for the increase in their cross-sectional area could make the transfer of well-perfused tissue to ischemic tissue clinically useful and are the subject of ongoing research in our laboratory.

This study is not truly quantitative. The new microvessels tend to develop in clusters, with other areas devoid of new microvessels. The sections were scanned, and the vessels were counted and measured in fields that were centered on the clusters of the microvessels. This finding of "hot spots" of angiogenesis is consistent with the pattern of angiogenesis that is found in breast cancer, as described by Weidner et al.[23,24] The technique of counting microvessels in such "hot spots" is similar to the technique that was described by these authors for staging breast cancers. This study allows a semiquantitative analysis of the temporal development of new vessels between the flap and the ischemic limb. The methods developed to stain, count, and measure microvessels in histologic sections of the flap-limb interface are being applied to computer-assisted microscope systems in our laboratory to allow a more quantitative assessment of the vessel number and cross-sectional area per unit area of the flap-limb interface. When refined, these computer-assisted techniques will enable quantitative comparison of various interventions used to enhance angiogenesis with this model.

Immunostaining of vascular structures in rabbit tissue is very difficult. Multiple commercially available antibodies to factor VIII–related antigen were tested and failed to selectively stain vascular endothelium. Various lectins also failed to differentiate blood vessels from the background tissues. The best selective immunostaining was achieved with antibodies to α-actin at dilutions of 1:150 and 1:200.

CONCLUSION

New microvessels can be demonstrated, by histologic evaluation, to develop at the interface between the muscles of the hind limb of a rabbit that was rendered ischemic by transection of the common iliac

artery and a well-vascularized muscle flap that is based on the contralateral iliac arterial system. PCNA immunostaining confirms that these are new, proliferating microvessels. At 7 days, these vessels have a median diameter of 8 μm and contain smooth muscle cells in the walls. At 14 days, the number of new vessels does not change, but the median diameter increases to 22 μm. Angiographic evaluation suggests that these new vessels provide connections between the arteries of the flap and the arteries of the hind limb. Studies on the effect of exogenous angiogenic factors to sustain the growth and development of these new blood vessels are in progress.

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DISCUSSION

Dr. Christopher K. Zarins (Palo Alto, Calif.). This is a very interesting study that addresses the question of whether nutritive blood flow could be supplied to an ischemic muscle by transferring and attaching to its surface a vascularized muscle flap. The authors have clearly demonstrated with angiographic and histologic examinations that vascular communication develops between the transposed muscle flap and the recipient bed. They suggest that ischemia is the driving force for the development of this connection, and they further suggest that these channels may convey nutritive blood flow to the ischemic tissue. A number of questions are raised by these observations.

The first relates to the vascular channels you identified in the interface between the two areas. What kind of vascular channels are these? Angiographic contrast shows a communication. Are they artery-to-artery connections? Artery-to-vein? What is the orientation of these channels? You cut sections perpendicular to the interface and selected circular channels. Therefore, they were parallel to the interface. Did they penetrate into the muscle on either side? You did serial sections. Did you do three-dimensional reconstruction and trace the channels? Do they communicate with the capillary bed?

Second, in regard to ischemia as the driving force of the process, was the model really ischemic? It is very difficult to actually make a limb ischemic. Did you use a Wood's lamp on the recipient rabbit? Did the histologic evaluation show ischemia? Did you do control studies, such as transposing the flap to a nonligated bed?

My third question relates to the concept of nutritive flow and whether these new vascular connections are artery-to-artery or arteriovenous. Is there any evidence that flow from the flap actually reaches the capillary bed of the recipient muscle? Could the flow from the recipient bed be helping to nourish the transposed flap at its edges with the most vulnerable blood supply?

My fourth question relates to your methods and statistics. This was a nonrandomized study. How did you select the magnification for the high-power field? How many fields did you count? What was the overall count of vessels? Were these vessels at the edge or the center of the interface? Statistically, this was a nonrandom sampling with only a few rabbits, two at 3 days, three at 7 days, and four at 14 days. How valid are your statistics? Are you comfortable with your conclusion that the channels enlarged? Could the larger 14-day channels be venules rather than arterioles?

Finally, as with all experimental studies, they often raise more questions than they answer, and I look forward to further published work with this model. Clinically, there is no question that transposed musculocutaneous flaps can successfully cover skin defects; however, there must be a suitable bed. I have always assumed that the vascular pedicle of the transposed flap primarily supplies the flap itself and ensures its survival. I am not convinced that it brings a new nutritive flow to the recipient bed and underlying muscle. This is, however, an important area for future investigation, and I am hopeful that it will be found.

Dr. William C. Pevec. Thank you, Dr. Zarins. You asked some very good questions, a lot of the same questions that we had on the basis of this model. As you point out, any basic science project usually ends up raising more questions than answers.

One of the parts of your first question was whether these are artery-to-artery connections or artery-to-vein connections. I cannot answer that question. That is a question that we are curious about, as well. I suspect that these new vessels probably are connecting into the arterial system. In a previous study[8] we injected microspheres into the arterial circulation of the rabbits. The results showed increased perfusion of the hind-limb muscles in rabbits that had a flap, as opposed to the control rabbits that did not have a flap. This finding suggests that the microspheres are getting into the arteries. We actually saw increased flow even all the way down into the calf, not just in the underlying muscle bed.

It would be expected that if these are arterial-to-venous connections, we would not see the microspheres down in the more distal portion of the limb. It would suggest indirectly that these are artery-to-artery connections, on the basis of the previous model.

The second part of your first question had to do with the orientation of the newly formed channels. You are absolutely correct. We did perpendicular sections, and the vessels we measured were ones that were cut on cross-section, but we saw vessels in virtually all orientations in those perpendicular sections.

So it is difficult to say exactly what was the direction of the blood vessels and the orientation between the flap and the underlying limb. It would be very helpful to examine these vessels with serial sectioning to find their ultimate connection. That's a very daunting histologic task that we have not done, but it would be the ultimate confirmation, anatomically, that the connections do exist.

Your second question involved the ischemic limb model of simply dividing the common iliac artery. I would totally agree with you that it's very difficult to make the limb of an animal ischemic. There have been multiple studies that looked at chronic ischemia models in the hind limb of various animals, and it's very difficult to do.

When we first were trying to develop this model, we performed multiple ligations along the iliofemoral tree in an attempt to induce ischemia. However, we settled on this model of simple division of the common iliac artery and actually published our work on this model, trying to show the level of ischemia, about 4 or 5 years ago.[9]

The index of the blood pressure of the ischemic ankle versus the contralateral, normally perfused ankle was significantly decreased. The index started out at 0 when compared with the contralateral limb at day zero and gradually recovered, but only to 0.5, as long as 38 days after the operation.

The second thing we looked at was arteriovenous oxygen difference, or oxygen extraction, of the control limb as compared with the experimental limb. We showed, out to 24 days, that there was increased extraction of oxygen from the limb that had the ligated artery, which would suggest a relatively hypoperfused limb.

The third method we used was microsphere injection. That method suggested that somewhere between about day 7 and day 17 the resting perfusion in the experimental limb approached 80% of that in a control limb.

It is difficult to put together the results of these various methods. It depends on what you call ischemia. Clearly these animals recovered. They develop collateral vessels, as the ischemia seems to be worse early on. The animals recover with time. It's not an absolute ischemia, and whether this is an ischemic limb depends on how you define ischemia. We would say that at least up to 14 days there is some relative ischemia of the limb with ligation of the common iliac artery as compared with a normal limb.

We did not examine the underlying limb with a Wood's lamp. That would be an interesting qualitative way to assess it. Again, with an early model we did do that. We actually used fluorescent monitoring to try to assess the degree of ischemia in limbs with various degrees of arterial ligations, and we did not find a huge difference between simple common iliac artery ligation and ligation of all the iliac and femoral branches, similar to a model that you described. That led us to just do a simple common iliac artery ligation.

You asked about comparing this model with, as a control, a model that did not use common iliac artery ligation. We actually performed those studies in the past and demonstrated with angiography that connections do appear to develop between the flap and the underlying limb muscle even if you don't do anything to render the limb ischemic. So this phenomenon does not seem to be entirely hypoxia-driven. There is clearly something more to the process, but that was not the focus of this current study.

Your third question involved finding the source of nutritive flow in this flap model and whether the underlying bed supplies the flow to the flap or vice versa. There is really no way, on the basis of this study, that we can say which of the two is the origin of these vessels, whether it's the underlying bed or the flap.

That is an important question that I don't think we can answer with this model. We showed that connections develop. Where they are originating is another question that we have been curious about.

A nice study[13] looked at implanting free muscle grafts into a hamster cheek pouch. They did very careful serial sectioning over half-day intervals, and they suggested that, in fact, the new blood vessels grew from the ischemic muscle of the graft, as opposed to from the well-vascularized bed, which would be the opposite of what one would assume. So that's a very good question.

Finally, you asked about our statistical methods. This study was performed with what is clearly a semiquantitative technique. This is not a quantitative technique, and we're working on trying to look at vessel number and vessel diameter across the whole area of the interface between the flap and the muscle. That's a project we're working on.

There is some precedent, however, for the technique of counting vessels just where they're clustered. It appears that angiogenesis occurs in so-called hot spots. Weidner et al.[23,24] published a couple of studies that looked at the prognosis of breast cancer on the basis of the number of angiogenic vessels in the specimen, and they reported that angiogenesis is not a homogenous process. There are some areas where there are clusters of the microvessels and large areas where there are no microvessels.

In their studies, Weidner et al. counted the number of vessels per 200× field centered on the clusters of microvessels and were able to show prognostic significance of the number of vessels counted in those hot spots. So our data by no means suggest that there is an absolutely greater number of vessels in one group versus the other group, but that the number of vessels per cluster does not change, while the vessel diameter does increase.

We counted about five sections per rabbit, so the numbers are not huge. The distribution, however, was nonparametric, so we thought the Mann-Whitney test would be the most appropriate one, and we were able to show significance for our small number of individual data points.

Could these be venules or arterials? It's impossible to answer that question directly on the basis of this model.

Dr. Alexander W. Clowes (Seattle, Wash.). I found this to be a very interesting study, and I had a couple of short questions for you.

First of all, is the ability to form these connections dependent on the age of the animal?

Second, have you developed any understanding about the molecular mechanism, either by adding in such things as VEGF or fibroblastic growth factor or blocking them and seeing whether these connections form?

Dr. Pevec. With regard to whether the effect is dependent on the age of the animal, we have not done that study. We have not looked at this process in younger animals versus older animals.

In the previous report to which I alluded, we used rabbits that weighed approximately 4 to 4.5 kg. In this study, we used rabbits that were of 2.5 to 3.5 kg weight, which suggests that these were younger animals. We have not specifically controlled for age, although certainly in these two populations the connections did appear to develop.

Determining the molecular mechanism of the formation of new vessels is really the goal of this study. My original hope was to somehow be able to perform an assay for angiogenic factors at this interface. That assay turns out to be a pretty difficult task, at least in the rabbit model.

Our next step is to add both VEGF and bFGF, both systemically and locally at the flap-muscle interface. I wanted to develop quantitative histologic techniques so that we could compare various dosages and various routes of administration of the angiogenic factors, so that we could see if we can augment this response.

If we're able to do that, then our next hope is to try to block the endogenous factors and see if they're playing a factor in this. So that is actually the goal. This is really a preliminary study for the very question you asked.

Dr. Max R. Gaspar (Long Beach, Calif.). You have been studying neovascularization in striated muscle. Have you performed any studies on smooth muscle? I'm thinking about the work that has been done in trying to get neovascularization in the myocardium.

Dr. Pevec. We have not. You probably are aware of Unger's technique of implanting the internal mammary artery into ischemic myocardium, suggesting that connections do, in fact, form and that they improve the perfusion in the ischemic region of the myocardium. We have chosen to look at this phenomenon in a skeletal muscle model, and we have not looked at any other models.

Dr. Foley. I recently had a situation where I performed a femoral-to-dorsalis pedis in situ saphenous vein bypass procedure for rest pain, a gangrenous toe, and a large

ulceration over the medial malleolus. In addition, a free flap was anastomosed to the dorsalis pedis bypass to cover the medial malleolar area, and it healed well. The bypass occluded 6 months later, and the free flap lived. This would suggest that nutrient flow into the flap from the surrounding tissues had developed. If nutrient flow is able to go from surrounding tissue into the free flap, then wouldn't nutrient flow be able to go the opposite direction? Does this lend support to your notion of new vessel ingrowth providing nutrient flow?

Dr. Pevec. I would suggest that the connections probably do form nutritive flow, on the basis of our earlier microsphere study that suggested that there was increased flow throughout the limb with this model.

This whole study was started on the basis of a clinical case, which was reported in the *Journal of Vascular Surgery* in 1990 by Ken Shestak from the University of Pittsburgh (Shestak KC, Hendricks DL, Webster MW. 1990;12:581-5). The reason we became interested in this topic was that we had a patient similar to yours who had an ischemic ulcer on the dorsum of his foot and had no reconstructible arteries in the distal leg. A free flap was placed over that ulcer, and was vascularized off of a saphenous vein graft from the suprageniculate popliteal artery. The free flap survived, but interestingly the patient reported a decrease in his symptoms of rest pain a couple of months after the operation.

A follow-up arteriogram was obtained, and it suggested filling of the native vessels with connections between the free flap and the underlying bed. There actually was better visualization of the plantar and pedal arteries in the foot, which could not be demonstrated previously, with antegrade flow indirectly suggesting that nutritive flow did come from the flap to the underlying muscle.

It is pretty clear from the plastic surgery literature that flow in the opposite direction happens. If you take a free vascularized flap and put it on a healthy bed and then ligate the inflow vessels after 3 or 4 weeks, usually that flap will survive, which suggests that a healthy bed will provide perfusion of a free flap.

There is some question, however, whether a free flap will supply blood to an ischemic bed. Our hypothesis is that it happens in both directions; that, in fact, you can get improvement in the ischemic bed. We're trying to nail it down more carefully with these studies.